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Platelet Physiology: In Cold Blood Dispatch

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One of the impediments precluding long-term storage of platelets for blood transfusion is that refrigerated platelets are rapidly cleared from the circulation upon transfusion. New evidence suggests that this clearance is mediated in the liver by the Mac-1 integrin on Kupffer cells recognizing clustered GPIb receptor on platelets, leading to platelet phagocytosis.

Platelets in the blood are the primary cells responsible for the control of bleeding. Under normal circumstances, their activation in response to bleeding triggers the clotting process; however, inappropriate activation leads to thrombosis, the precipitating event in heart disease and stroke. In contrast to other blood components, platelets have a limited shelf-life for transfusion purposes of 5 days, since they must be stored at room temperature and are thus prone to microbial contamination. This method of storage is necessary because refrigeration of platelets, even for short periods, leads to their rapid clearance from the circulation upon transfusion. A recent study sheds light on the mechanism underlying this phenomenon. Interestingly Hoffmeister and colleagues [1] now demonstrate that clusters of the glycoprotein (GP)Ib alpha subunit of the platelet adhesion receptor GPIb-IX-V are recognised by the Mac-1 integrin and this interaction is responsible for the clearance of chilled platelets upon transfusion. These findings suggest that GPIb-targeted approaches might permit cold platelet storage providing increased utility and substantial savings in healthcare cost.

Although platelets normally circulate freely, they can rapidly adhere to exposed breaks in the endothelium and to activated endothelial cells. Such adherent platelets are rapidly activated leading to platelet aggregate formation through upregulation of the platelet-specific integrin, GPIIb-IIIa and its subsequent binding to adhesive ligands such as fibrinogen and von Willebrand factor (vWF) [2,3]. vWF is also a key protein mediating the initial platelet adhesion, via an interaction with a unique multisubunit platelet adhesion receptor, GPIb-IX-V.

GPIb-IX-V consists of four type I membrane-spanning subunits, all of which are members of the leucine-rich repeat protein superfamily. GPIbα is disulfide-linked to GPIbβ, and these subunits are non-covalently associated with GPIX and GPV in the ratio of 2:2:2:1 [2,3]. The extracellular amino-terminal domain of GPIbα (282 residues) contains the primary ligand recognition sequences and is separated from the membrane by a sialomucin core. In resting platelets, the cytoplasmic sequence of GPIbα (~100 residues) provides a binding site for filamin A and filamin B. It is thus the major membrane glycoprotein linked through filamin to a network of short, submembranous actin filaments [4,5]. The penultimate residue (Ser609) at the carboxyl terminus of GPIbα is constitutively phosphorylated allowing binding of 14-3-3ζ and the assembly of other signalling molecules such as phosphatidylinositol 3-kinase and Src [2]. The cytoplasmic domain of GPIbβ (~34 residues) contains an additional binding site for 14-3-3ζ that requires protein kinase A (PKA)-mediated phosphorylation at Ser166 and is adjacent to an upstream positively charged motif that binds calmodulin [6,7]. Calmodulin can also bind to the cytoplasmic tail of GPV [7].

The binding site for vWF is present in the amino-terminal domain of GPIbα (residues 1–282). This consists of an amino-terminal flanking sequence, eight leucine-rich repeats, a carboxy-terminal flanking sequence and an anionic sequence containing three sulfated tyrosines (Figure 1). The interaction of GPIb-IX-V with vWF is shear dependent and involves a specific segment in vWF, the A1 domain. A-domains are found in many adhesive proteins, including integrins such as Mac-1, where they are present as an insert sequence in the α-chain of the integrin. An insight into how GPIbα and vWF interact has recently been provided by the X-ray crystal structure of a gain-of-function A1 domain fragment of vWF in complex with a gain-of-function fragment of the amino-terminal domain of GPIbα (the gain-of-function mutation in each fragment facilitates complex formation) [3,12,13]. Binding involves contact of the A1 domain by residues in the amino- and carboxy-terminal flanking sequences of

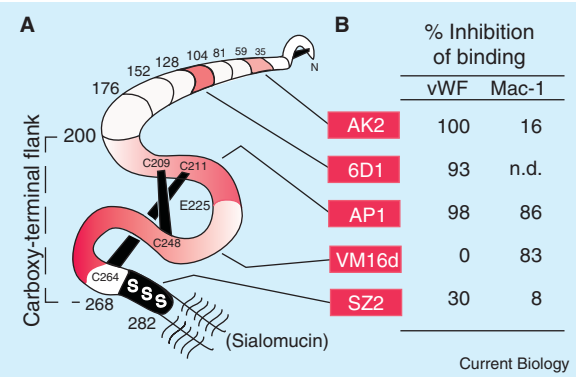

Figure 1. The amino-terminal domain of GPIbα.

(A) Schematic representation of the amino-terminal domain of GPIbα (residues 1–282) consisting of the leucine-rich repeats (including the sequence 35–200) and their disulfide-looped capping sequences. Sulfated tyrosine residues at 276, 278 and 279 are within an anionic sulfated sequence 269–282, defined by the mocarhagin cleavage site at 282/283. Epitopes for five anti-GPIbα monoclonal antibodies are indicated [8–10]. (B) Function of anti-GPIbα antibodies in inhibiting GPIbα binding to either von Willebrand factor (vWF) with ristocetin [8–10], or to Mac-1 [11]. The glycopeptide antibiotic ristocetin activates vWF to bind GPIbα *in vitro* and most accurately reflects the shear-dependent interaction [10].

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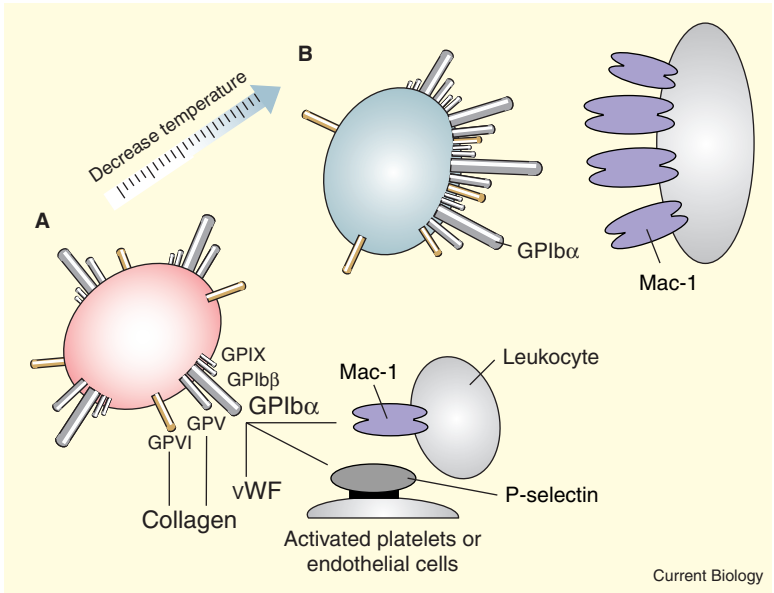

Figure 2. Interactions of platelet GPIb-IX-V.

(A) Platelet adhesion is mediated by receptors, GPIb-IX-V and GPVI, that bind von Willebrand factor (vWF) or collagen. GPIbα binds vWF in subendothelial matrix or plasma, P-selectin on activated platelets or endothelial cells, and leukocyte Mac-1. GPV and GPVI interact with collagen. All these interactions may activate platelets, leading to aggregation mediated by the integrin GPIIb-IIIa. (B) At lower temperatures, clustering of GPIbα is implicated in clearance of platelets from the blood by a mechanism involving hepatic macrophage Mac-1 [1]. The temperature effects on surface topography of other platelet receptors such as GPVI are unknown.

GPIbα, as well as electrostatic interactions involving the leucine-rich repeats (residues 60–128) [3,8,13]. Most notable is a major conformational change in the carboxy-terminal flanking sequence, the β-switch, allowing an extended β-sheet involving both receptor and ligand [13].

GPIb-IX-V has additional roles in vascular biology. The sulfated tyrosine sequence (Figure 1) provides a binding site for α-thrombin that facilitates platelet activation through the thrombin receptors, PAR-1 and PAR-4 [2]. GPIbα binds P-selectin, an interaction involved in platelet translocation on activated endothelium (Figure 2) [2,14]. Finally, Simon and coworkers demonstrated that GPIbα was the primary recognition site on platelets for the leukocyte integrin, Mac-1 (αMβ2; CD11b/CD18; CR3) [2,11], via an interaction between the inserted A domain (I domain) of Mac-1 and the amino-terminal domain of GPIbα. Mac-1 is the primary integrin present on monocytes/macrophages, and is responsible for mediating phagocytic events. It binds multiple ligands including the complement fragment iC3b, and the cell adhesion molecule ICAM-1. Interestingly, although the interaction of GPIbα with both vWF and Mac-1 involves binding of homologous A-domains, antibody-blocking experiments (Figure 1) suggest the binding sites are in part distinct, with Mac-1 recognition primarily involving the GPIbα carboxy-terminal flanking sequence.

There is evidence to suggest that the interaction between GPIbα and Mac-1 may provide a mechanism for monocyte/macrophage transmigration through thrombi during the response to injury in vascular repair [15]. Hoffmeister *et al.* [1] have now described in *Cell* an additional physiological role for this interaction, namely in the clearance of cold-treated platelets. It has long been recognized that exposure of platelets to temperatures below 15°C leads to a change in shape and this has been widely believed to be the cause of their accelerated clearance. Both increased cytoplasmic Ca^{2+} concentration and membrane phosphoinositide clustering have been demonstrated to be causes for cold-induced actin filament rearrangement and platelet rounding [16,17]. Hoffmeister *et al*. [1] surprisingly found that, although treatment of platelets with the membrane-permeable Ca^{2+} chelator, EGTA-AM, and the actin filament barbed-end capping agent, cytochalasin B, kept them discoid on cooling [17], these platelets were still rapidly cleared [1].

If platelet shape is therefore not critical for cold-induced clearance, how is it occurring? Hoffmeister *et al.* [1] made the key observation that cold-treated mouse platelets are cleared in the liver by Kupffer cells (sinusoidal macrophages), but that this did not occur in Mac-1-deficient mice where the platelets were cleared at the same rate as platelets held at room temperature. The integrin Mac-1 is thus critical for the clearance of cold-treated platelets, inferring a potential role for GPIbα as the key element on platelets involved in this phenomenon. Indeed, proteolytic removal of GPIbα from mouse platelets prevented their clearance following cold treatment. Similarly, selective removal of GPIbα on cold-exposed human platelets by a snake venom metalloproteinase, mocarhagin [18], prevented their Mac-1-dependent phagocytosis by activated THP-1 cells [1]. Some irreversible change clearly must occur in GPIbα at low temperatures allowing its increased recognition by Mac-1. Hoffmeister *et al.* [1] found that chilling led to irreversible clustering of GPIbα on mouse platelets as revealed by immunogold localization, although whether this is the direct cause for enhanced Mac-1 binding remains to be established.

A remarkable finding from this study is that, even though cold-treated platelets are rapidly cleared, they are inherently hemostatically effective [1]. For example, cold treatment does not affect the capacity of GPIbα to bind vWF, suggesting that the changes in GPIbα recognized by Mac-1 are distinct from GPIbα recognition of vWF. Cold treatment does not lead to surface expression of the α-granule activation marker, P-selectin, or upregulation of the function of the GPIIb-IIIa

integrin. Circulating chilled platelets have normal hemostatic function in Mac-1-deficient mice, and form platelet aggregates at a wound site to a similar extent as endogenous platelets.

These combined findings raise the intriguing possibility that GPIbα could be modified in such a way that cold platelet storage may be feasible with normal hemostatic effectiveness and without accelerated clearance. The obvious approach would be to prevent the GPIbα clustering and/or conformational changes that occur on cooling platelets, but this will require additional molecular understanding of these phenomena. One outstanding issue is how clustering of GPIbα occurs at low temperature. Since clustering still occurs in the presence of Ca^{2+} chelation and cytochalasin B [1], mechanisms other than actin filament rearrangement must be involved. 14-3-3ζ is dimeric suggesting it could be involved in a receptor multimerization process; studies have shown that deletion of the carboxy-terminal 14-3-3ζ binding site from GPIbα markedly increases receptor motility [19]. Calmodulin is known to dissociate from GPIb-IX-V on platelet activation [7], and this event could trigger receptor aggregation. A third possibility is that receptor clustering may involve only a subpopulation of GPIb-IX-V. In this regard, Shrimpton *et al.* [20] have reported a subpopulation of GPIb-IX-V receptors in cholesterol-rich membrane rafts. There is also the intriguing possibility that there may be an irreversible conformational change in the carboxy-terminal flanking sequence of GPIbα. This region is directly implicated in Mac-1 binding [11], and is capable of undergoing dramatic changes in conformation [13].

Finally, why have we developed a clearance mechanism that only appears to operate for cold-activated platelets? Hoffmeister *et al.* [1] speculate that since platelets show increased responsiveness to activation as the temperature falls below 37ºC, they may act as thermosensors '*designed to be less responsive to thrombogenic stimuli at the core body temperature of the central circulation where coronary or cerebral thrombosis could be lethal, but become primed for activation at the lower temperatures of external body surfaces, sites most susceptible to bleeding throughout evolutionary history*'. Consistent with this speculation, they show that normal mice but not Mac-1-deficient mice have decreased platelet counts after 2 hours of exposure to 4ºC, relative to the corresponding mice at room temperature. Clearance of platelets by liver Kupffer cells may thus provide a physiological mechanism for removal of more activated platelets primed by repeated exposure to lower temperature in the peripheral circulation.

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